Epitope analysis of antibodies recognising the cell proliferation associated nuclear antigen previously defined by the antibody Ki-67 (Ki-67 protein)

M H G Kubbutat, G Key, M Duchrow, C Schlüter, H-D Flad, J Gerdes

Abstract

Aims—To elucidate the fine specificities of the antibodies MIB 1 and MIB 3 and of additional monoclonal antibodies which also recognise the Ki-67 protein (MIB 5, IND.64, JG-67-2a).

Methods—Different parts of the Ki-67 protein cDNA were expressed in *Escherichia coli*. Bacterial lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on to nitrocellulose. Additionally different peptides were synthesised on a membrane support (SPOT-Blot). The immunoreactivity of the antibodies with the recombinant proteins and the immobilised synthetic peptides, respectively, was analysed. A competition enzyme linked immunosorbent assay (ELISA) using a soluble synthetic peptide was also performed.

Results—The epitopes of all antibodies tested were contained within the same region of seven amino acids. The antibodies MIB 1 and MIB 3 required the five amino acid sequence FKELF for binding, whereas Ki-67, JG-67-2a, MIB 5 and IND.64 detected the sequence FKEL.

Conclusions—It is concluded that the amino acid sequence FKELF represents an immunodominant area of the Ki-67 protein and that there is no correlation between the ability to detect the Ki-67 protein in paraffin wax sections irradiated with microwaves and the epitopes recognised by the antibodies.

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Forschungsinstitut Borstel, Division of Molecular Immunology, Parkallee 22, D-23845 Borstel, Germany
M H G Kubbutat
G Key
M Duchrow
C Schlüter
H-D Flad
J Gerdes
Correspondence to:
Dr J Gerdes

The monoclonal antibody Ki-67 was found in 1983 after mouse myeloma cells had been fused with the spleen cells of mice immunised with nuclei of L 428 cell line cells.[1] Detailed cell cycle analysis showed that this antibody detects a nuclear antigen (Ki-67 protein) which is exclusively expressed in proliferating cells—that is, in G_1, S, G_2 phases, and mitosis, but not in G_0.[2]

Because of these properties, Ki-67 has become a powerful tool during the past decade for estimating the growth fraction in malignant disease, based on numerous studies which have consistently shown that there is a highly significant correlation between the mean values of the growth fraction deter-mined with the monoclonal antibody Ki-67 and the histopathological grade of malig-nancy.[3–5]

The inherent drawback of Ki-67 is that it can be applied only to fresh material, and not routinely formalin fixed and paraffin wax embedded tissue—and, therefore, in retro-spective studies of archival material. Recently, we produced new monoclonal antibodies using a recombinant partial structure of the Ki-67 protein as immunogen. These antibod-ies, designated MIB 1–3, represent true Ki-67 equivalents as determined by histochemistry and immunobiochemistry.[6]

Two of these new antibodies (MIB 1 and MIB 3) as well as the antibody MIB 5 gener-ated subsequently[7] can detect the Ki-67 pro-tein in routinely formalin fixed, paraffin wax embedded normal and tumour tissues when using an antigen retrieval method based on microwave treatment.[8 9] Thus the new Ki-67 equivalent antibodies MIB 1, 3, and 5 now permit the detection of the Ki-67 protein on archival material.

First studies showed that the epitopes of MIB 1, MIB 3, and Ki-67[6] as well as that of MIB 5 (unpublished results) are located on an identical stretch of 20 amino acids within the Ki-67 protein. This sequence represents a part of the highly conserved 22 amino acid element called "Ki-67 motif". In the Ki-67 protein it appears 16 times with a homology between 72% and 100% with regard to the amino acid sequence (fig 1A).[10]

Because of the importance of estimating the Ki-67 growth fraction, the aim of our study was to elucidate the fine specificities of antibodies directed against the Ki-67 protein within the 22 amino acid sequence mentioned above, and to compare the epitopes recognised by the antibodies MIB 1, 3, 5, Ki-67, and JG-67-2a[11] and the recently described monoclonal antibody IND.64,[12] which represents an addi-tional Ki-67 equivalent antibody.[13]
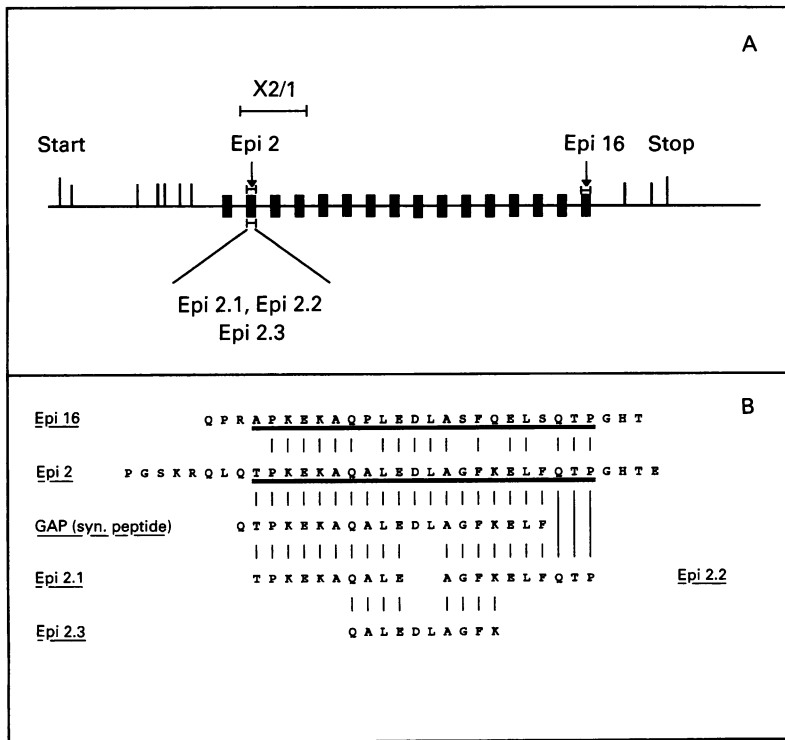
Methods

All amino acid sequences mentioned are writ-ten in the one letter code from the N-terminus to the C-terminus (from left to right).

Antibodies MIB 1, 2, 3, and 5, Ki-67 and IND.64 were generated as described[1 6 7 12]; JG-67-2a is a subclass switch variant of the original Ki-67 antibody.[11] Gal 1 is a monoclonal anti-β-galactosidase antibody produced in our laboratory.

Figure 1 *A: Schematic structure of the Ki-67 protein cDNA. The positions of the cloned sequences are indicated. Marked boxes represent the 16 repeats called "Ki-67 motifs" which show a homology between 72% and 100% with regard to the corresponding amino acid sequence. B: Amino acid sequences used in this study. Sequence Epi 2, Epi 16, Epi 2.1, Epi 2.2 and Epi 2.3 were expressed as part of fusion proteins in E coli, whereas the peptide GAP was synthesised and used in a competition assay by ELISA. Double underlined sequences represent the "Ki-67 motifs"; single lines mark positions of identical amino acids.*

CONSTRUCTION OF PLASMIDS

Nucleic acid sequences encoding for amino acid sequences of Epi 2 and Epi 16 (figs 1A and B), respectively, derived from the Ki-67 protein cDNA, were amplified from IM-9 cDNA (ATCC No. CCL 159) using the polymerase chain reaction (PCR). The fragments were cloned between the *Eco* RI and *Bgl* II site of the expression vector pAX 4a + [14] (Medac, Hamburg, Germany). The resulting plasmids were transformed into *E coli* DH5αIQ (Gibco BRL, Berlin, Germany).

Sense and antisense strands of the sequences encoding for Epi 2.1, 2.2, and 2.3 (figs 1A and B) were synthesised on a Beckman DNA-SM Synthesizer (Beckman, Munich, Germany) with additional bases on the 5' and 3' end to form restriction sites after hybridisation. The corresponding sense and antisense strands were hybridised by heating for 10 minutes to 95°C and slowly cooling down to room temperature. The double strand fragments were separated on non-denaturing PAGE and eluted from the gel by the "crush and soak" method.[15] The purified fragments were cloned between the *Bgl* II and *Sal* 1 site of the pAX 4a+ vector and transformed into *E coli* DH5αIQ.

All resulting plasmids carried a gene which encodes for a fusion protein containing bacterial β-galactosidase and the respective part of the Ki-67 protein.

SEQUENCING OF INSERTS

Plasmids of successfully transformed *E coli* clones were isolated using Qiagen Tip-100 columns (Diagen, Hilden, Germany). Sequencing of purified plasmids was performed on an ALF DNA sequencer according to the manufacturer's recommendation (Pharmacia, Freiburg, Germany).

SDS-PAGE AND IMMUNOBLOTTING

Preparation of bacterial cell lysates for SDS-PAGE was performed as described.[6] Lysates of bacteria were separated on reducing 7·5% separation gels with 3% stacking gels. Molecular weight markers were obtained from Sigma (Munich, Germany). Proteins were transferred to nitrocellulose membranes (BA 85, Schleicher & Schuell, Dassel, Germany) by western blot technique[16] in a Biometra (Göttingen, Germany) semi-dry blotting apparatus (two hours at 150 mA).

Unoccupied protein binding sites of the nitrocellulose were blocked by incubation for one hour with blocking solution (2% bovine serum albumin (BSA), 0·05% Triton X-100 in TRIS-buffered saline (TBS)). After one hour of incubation with primary antibody and thorough washing with TBS the bound antibody was targeted with goat anti-mouse alkaline phosphatase conjugate (Dianova, Hamburg, Germany), diluted 1 in 10 000 in blocking solution.

The phosphatase activity was visualised using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate.[17]

SPOT-BLOT

Solid phase peptide synthesis on a special membrane support (SPOT-Blot) was performed according to the manufacturer's instructions for the membrane (ICI, Cambridge Research Biochemicals, Norwich, England).

Blocking of unoccupied protein binding sites, incubation, and detection of bound antibodies were performed as described above. Synthesised peptide sequences are shown in fig 5.

COMPETITION ASSAY BY ELISA

Competition assay by ELISA was performed as described before.[6] Briefly, polystyrene microtitre plates were coated with lysates of bacteria expressing a part of the Ki-67 protein cDNA (X2/1) (fig 1A), known to include the epitopes of all antibodies tested.[6 7 13] Antibodies were incubated with different concentrations of the synthetic peptide, designated GAP (fig 1B), for 30 minutes before being subjected to precoated microtitre plates. After incubation for one hour bound antibodies were detected using a goat anti-mouse alkaline phosphatase conjugate in combination with a phosphatase substrate (Sigma, Munich, Germany). Finally, optical density was determined at 405 nm.

Synthetic peptide GAP was synthesised by Dr Kullmann, Institut für Neurobiochemie, Hamburg, Germany.

Results

All cloned inserts used in this study were fully

Summary of biochemical and immunohistochemical properties of the Ki-67 equivalent antibodies used in this study

| Antibody | Subclass | Immunogen | Immunohistochemical detection of the Ki-67 protein on | | Western blot | | Reference |
			Frozen sections	Microwave treated paraffin wax sections	Native Ki-67 protein (IM-9 lysate)	X 2/1	
Ki-67	IgG 1 κ	Nuclei of L 428 cell line cells	+¶	–	+	+	1, 6
JG-67-2a	IgG 2a κ	φ§	+	–	+	+	11, unpublished data
MIB 1	IgG 1 κ	X2/1†	+	+	+	+	6
MIB 2	IgG 1 κ	X2/1	+	–	+	+	6
MIB 3	IgG 1 κ	X2/1	+	+	+	+	6
MIB 5	IgG 1 κ	X2/1	+	+	+	+	7
IND-64	IgG 2b κ	Spleen cells of nude mice grafted with Ichikawa tumor	+	–	+	+	12, 13

§JG-67-2a is a subclass switch variant of the original Ki-67; †X2/1 is a recombinant fusion protein containing β-galactosidase and a part of the Ki-67 protein; ¶+ reacts with; – no reaction.

sequenced and controlled with regard to the reading frame and the sequence.

COMPARISON OF IMMUNOHISTOCHEMICAL AND BIOCHEMICAL CHARACTERISTICS OF ANTIBODIES INVESTIGATED

The table summarises the immunohistochemical and biochemical data of the Ki-67 equivalent antibodies used in this study. All antibodies except JG-67-2a (IgG 2aκ) and IND.64 (IgG 2bκ) are of the IgG 1κ subclass. The immunogen of MIB 1, 2, 3 and 5 was a recombinant fusion protein including a part of the Ki-67 protein, designated X2/1. It contains three of the 16 repeats ("Ki-67 motifs") occurring in the Ki-67 protein (fig 1A) and was applied for immunisation after complete denaturation of the protein. The immunogen for the generation of the original monoclonal antibody Ki-67 consisted of isolated nuclei of L428 cell line cells; IND.64 was produced using spleen cells of nude mice grafted with Ichikawa tumour as immunogen. On frozen sections of proliferating tissues, such as human tonsils, all antibodies show an identical staining pattern. On the other hand, only MIB 1, 3, and 5 can stain proliferating cells in formalin fixed, paraffin wax embedded tissue sections after microwave treatment. In western blots all antibodies stain the Ki-67 protein in IM-9 cell lysates as well as the recombinant part of the Ki-67 protein (X2/1).

IMMUNOREACTIVITY OF ANTIBODIES WITH BACTERIALLY EXPRESSED PROTEINS DEDUCED FROM THE KI-67 PROTEIN cDNA

Monoclonal antibody MIB 2 detects an epitope which is localised within the sequence X2/1 except that part which is named Epi 2 (fig 1A).[6] Its epitope is not further characterised. The monoclonal antibody MIB 2 served as negative control in all experiments performed during this study, as we concentrated our analysis on sequence Epi 2. Accordingly, the monoclonal antibody Gal 1 recognising the β-galactosidase protein was used as positive control for the expression of fusion protein. Thus negative results obtained with MIB 2 and positive results of Gal 1 on western blots of bacterial lysates, respectively, are not mentioned further.

Figure 2 summarises the results obtained with the different Ki-67 equivalent antibodies applied on western blots of bacterial lysates after expressing the "Ki-67 motif" sequences Epi 2 and Epi 16, respectively. All antibodies detect the fusion protein containing the sequence Epi 2, whereas no antibody binds to the Epi 16 sequence.

COMPETITION ASSAY BY ELISA

The binding of all Ki-67 equivalent antibodies to the recombinant fusion protein β-galactosidase/X2/1 was inhibited by the synthetic peptide GAP (fig 3). This result is well in line with those obtained with the recombinant fusion protein (β-galactosidase/Epi 2) as described above (fig 2).

IMMUNOREACTIVITY OF ANTIBODIES WITH BACTERIALLY EXPRESSED PEPTIDES DEDUCED FROM KI-67 PROTEIN cDNA

To elucidate further the epitopes of the antibodies within the Epi 2 sequence, we constructed three plasmids which encode for three different fusion proteins. The fusion proteins consist of β-galactosidase and one of three different but overlapping 10 amino acid sequences (Epi 2.1, 2.2, and 2.3), each representing a partial sequence of Epi 2 (fig 1B). As shown in fig 4, all antibodies exclusively recognise the fusion protein containing the Epi 2.2 sequence, but not those including the Epi 2.1 or Epi 2.3 sequence. The results shown in figs 2, 3, and 4 show that all antibodies react with the Epi 2 and GAP sequence as well as with the Epi 2.2 sequence, but not with Epi 16, Epi 2.1, and Epi 2.3. Thus we assumed the seven amino acid sequence AGFKELF to be the region in which the epitopes of the antibodies are located, as this sequence appears in all constructions which lead to a positive reaction of the antibodies.

SPOT-BLOT

To localise the epitope within the sequence AGFKELF we synthesised six different overlapping heptapeptides (fig 5A) on a membrane support. With these different peptides we covered the amino acid sequence DLAGFKELFQTP. Because we could
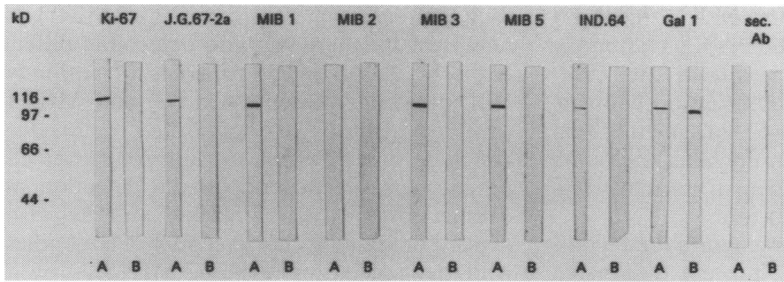
Figure 2   Immunoblot analysis of recombinant parts of the Ki-67 protein with different Ki-67 equivalent antibodies. Lane A: bacteria lysates after expression of β-galactosidase/Epi 2 fusion protein; lane B: as lane A, but with sequence Epi 16.
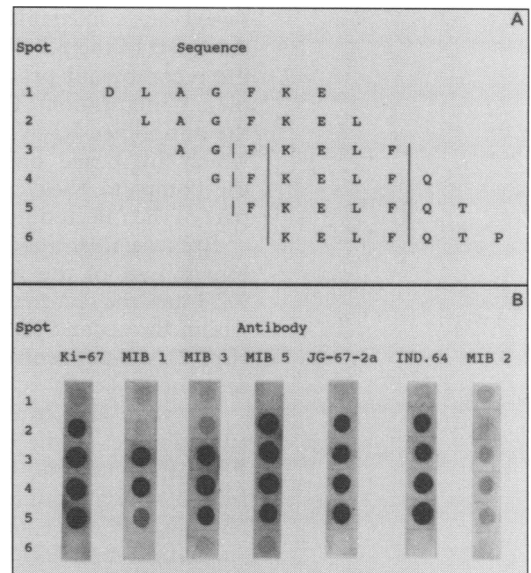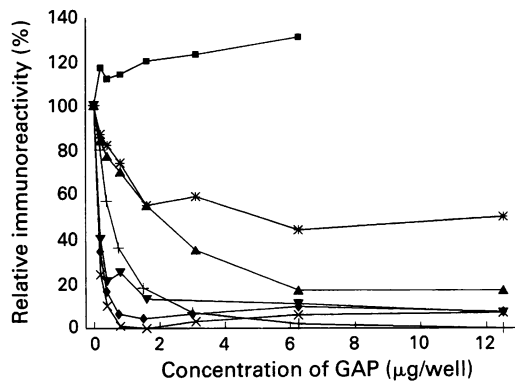

Figure 5   A: Sequences of the six different overlapping heptapeptides used in SPOT-Blot analysis as synthesised on a membrane support. Lines indicate consensus sequence of peptides recognised by monoclonal antibody Ki-67, MIB 5, JG-67-2a and IND.64; dashed lines indicate consensus sequence of peptides recognised by monoclonal antibody MIB 1 and MIB 3; B: Analysis of different overlapping heptapeptides derived from the Ki-67 protein cDNA for binding of different Ki-67 equivalent antibodies (SPOT-Blot analysis). Monoclonal antibodies Ki-67, JG-67-2a, MIB 5 and IND.64 detect only peptides including the four amino acid sequence FKEL; MIB 1 and MIB 3 detect only those including the amino acid sequence FKELF. MIB 2 was used as negative control. Solid lines mark consensus sequence for Ki-67, JG-67-2a, MIB 5 and IND.64; dashed lines enclose consensus sequence for MIB 1 and MIB 3.

exclude that the antibody MIB 2 recognises any sequence used in the SPOT-blot (see above), only those signals were regarded as positive which showed a stronger intensity than those obtained with the antibody MIB 2.

Figure 5 B shows that the antibodies Ki-67, JG-67-2a, MIB 5, and IND.64 detect only those peptides that include the four amino

Figure 3   Results of competition assay by ELISA using synthetic peptide GAP. (▼: Ki-67, +: JG-67-2a, X: MIB 1, ■: MIB 2,X: MIB 3, ◆: MIB 5, ▲: IND.64) The binding of all antibodies except MIB 2 to the solid phase-bound fusion protein β-galactosidase/X2/1 was inhibited by the synthetic 20 amino acid peptide designated GAP.

acids sequence FKEL (spot Numbers 2, 3, 4 and 5), whereas the antibodies MIB 1 and MIB 3 react only with those containing the sequence FKELF (spot numbers 3, 4 and 5).

Discussion

For nearly one decade the monoclonal antibody Ki-67 remained a unique reagent. Due to the fact that we succeeded in sequencing the cDNA of the Ki-67 protein,[7] a set of antibodies was raised against a recombinant part of the Ki-67 protein (MIB 1, 2, 3, and 5).[6] Another Ki-67 equivalent antibody (IND.64) was found by using splenocytes of mice grafted with Ichikawa tumour for somatic cell fusion. Some of the new antibodies might gain the same importance for immunohistopathological purposes as the original Ki-67 antibody, because they overcome the drawback that the application of Ki-67 is limited to fresh material (MIB 1, 3, and 5) and may permit double immunostaining due to different antibody subclasses (IND.64), respectively. Hence, the aim of our study was to elucidate the fine specificities of antibodies that recognise the cell proliferation associated nuclear antigen previously defined by the monoclonal antibody Ki-67.

The antibodies investigated can be divided into two groups with regard to the immunogens used for their generation. The first group includes antibodies (MIB 1, 2, 3, and 5)
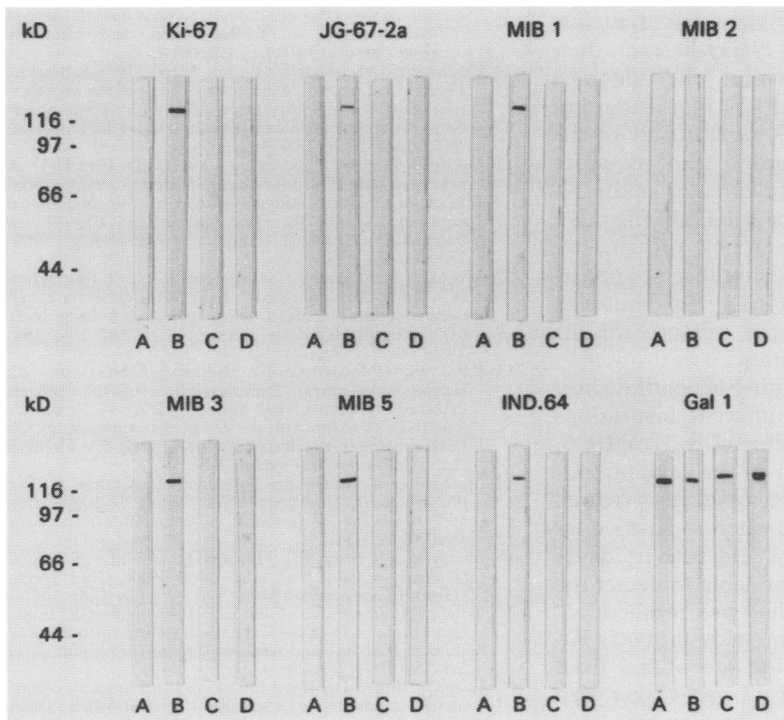

Figure 4   Immunoblot analysis of recombinant peptides with different Ki-67 equivalent antibodies. Lane A: bacteria lysates after expression of β-galactosidase/Epi 2.1 fusion protein; lane B: as lane A, but with sequence Epi 2.2, lane C: as lane A, but with sequence Epi 2.3, lane D: as lane A, but without any part of the Ki-67 protein sequence.

raised after immunisations with a recombi-
nant part of the Ki-67 protein (X2/1); notably
the recombinant protein was used after com-
plete denaturation. The other group contains
the antibodies Ki-67, JG-67-2a, and IND.64
which are based on immunogens including
the complete Ki-67 antigen in its native (as
part of the injected nuclei) form.

Because all antibodies recognise the recom-
binant part of the Ki-67 protein designated
X2/1 and the fact that some detect a sequence
within the second repeat of the Ki-67 protein
(Epi 2), we concentrated our analysis on this
sequence.

We were able to elucidate the epitopes of
all antibodies to the five amino acid sequence
FKELF. In contrast to antibodies MIB 1 and
MIB 3, which required all five amino acids for
binding, the other antibodies (Ki-67, JG-67-
2a, MIB 5 and IND.64) also react with the
four amino acid sequence FKEL. Hence all
Ki-67 equivalent antibodies show remarkable
conformity with regard to their epitopes, obvi-
ously regardless of the immunogen used for
their generation. The amino acid sequence
FKELF occurs in nine of 16 repeats (each
with a length of 22 amino acids) of the Ki-67
protein. These repeats show homologies
between 72% and 100% concerning their
amino acid sequence. Comparing the results
obtained with the Epi 2 and the Epi 16
sequence (two of these repeats), it becomes
clear why no antibody reacts with Epi 16,
although these two "Ki-67 motifs" have an
overall homology of 77%. The sequence
AGFKELF of Epi 2 corresponds to an area in
Epi 16, where only four homologous amino acids
appear (A-F-EL-), which are also separated
by non-homologous amino acids (fig 1B).

The different Ki-67 equivalent antibodies
investigated in this study were raised against
different immunogens. Nevertheless, their
epitopes are located within the identical
amino acid sequence FKELF, indicating that
this area is an immunodominant part of the
Ki-67 protein. As the amino acid sequence
EDLAG, which is located directly at the N-
terminus of the sequence FKELF (fig 1B),
appears 11 times in the Ki-67 protein, we
conclude that not only the number of appear-
ances of the sequence FKELF (9x), but also
its structure may be the reason for its
immunodominant properties.

In previous studies with histopathological
material obtained from different institutions
we showed that antibodies MIB 1, MIB 3,
and MIB 5 stain the Ki-67 protein in rou-
tinely processed, paraffin wax sections treated
by microwave irradiation, whereas Ki-67 does
not (unpublished data).[6 8 9] Recently, Cuavas
et al reported that they were able to detect the
Ki-67 protein in paraffin wax embedded,
microwave treated material with antibody Ki-
67.[18] However, the staining of optimal fixed
tissues by the antibody Ki-67 was weaker than
that of MIB 1 in less well fixed tissues.[18] As
there were no differences in staining tech-
nique or microwave treatment, we assumed
that binding of Ki-67 to its epitope seems to
be sensitive to variations in fixation or embed-

ding protocols whereas binding of MIB 1 is
not. Because there were no detectable differ-
ences in the staining properties of antibody
MIB 5 (same epitope as Ki-67) and MIB 1
(different epitope) (unpublished data), we
conclude that different staining properties do
not neccessarily depend on the detection of
distinct epitopes (Ki-67/MIB 1), but may also
depend on binding characteristics of antibodies
with identical epitopes (Ki-67/MIB 5).

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